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Lanthanide Enhanced Luminescence (LEL) with one and two photon excitation of Quantum Dyes® Lanthanide(III)-Macrocycles

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ABSTRACT

Improvements in the lanthanide enhanced luminescence (LEL) protocol have facilitated the use of the recently synthesized Eu(III)-macrocycle-mono-isothiocyanate, Quantum Dye®, as a label. It was discovered that a homogeneous solution in ethanol or other solvent could be used to produce the lanthanide enhanced luminescence (LEL) effect, provided that the solution was permitted to evaporate. This protocol has been applied to the direct staining of cells in S phase, and was optimized for solid phase assays with Quantum Dye labeled streptavidin. Preliminary studies indicate that cells stained with the europium Quantum Dye can be observed both by conventional UV laser excitation and by infrared two-photon confocal microscopy. An enhancer has been found that enables the observation of simultaneous emissions from both the europium and terbium Quantum Dyes.

Keywords: Luminescence, macrocycle, lanthanide, europium, terbium, digital microscopy, LEL, cofluorescence, isothiocyanate, solid phase.

1. INTRODUCTION

Much of the initial work on lanthanide enhanced luminescence, LEL, involved measurements of luminescence in aqueous micellar solutions[1,2,3]. A previous attempt[1] at performing luminescence measurements in an ethanol solution had resulted in an unacceptable decrease of the emission intensity. However, recent studies aimed at facilitating the present solid phase and cell monolayer work have shown that a homogeneous solution in ethanol or other solvent can be used to produce the LEL effect provided that the solution is permitted to evaporate. The resulting amorphous mixture containing the organic-biological materials, the lanthanide macrocycle label, an organic enhancer, and a second lanthanide ion appears to have some properties similar to lanthanide containing phosphors[4,5]. Yet, this mixture is suitable for standard histo- and cyto-chemical procedures including one and two photon confocal microscopy.

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2. METHODOLOGY

2.1. Chemicals and Disposables

The following were obtained from Sigma, (St. Louis, MO): PEG 1,450, polyethylene glycol with average mol. wt. 1,450 (product number P-5402), and 4,4,4-Trifluoro-1(2-thienyl)-1,3-butanedione (thenoyltrifluoroacetone, HTTFA) (product number T2,700-6). EuMac-Streptavidin and EuMac-di-NCS were obtained from Newport Instruments (San Diego, CA). Streptavidin was obtained from Prozyme (San Leandro, CA, part no. SA10). The following were obtained from Phoenix Flow Systems (San Diego, CA): Fluorescein labeled streptavidin (part no. Fluorescein-Strep), EuMac-Anti-5-BrdU (part no. Eu-PRB1), HL60 (non-apoptotic) cells (APO-BRDU™ Kit, part no. CC1001), and components from the ABSOLUTE-S™ Kit. The following components of the ABSOLUTE-S™ kit were used: 5-BrdU tailed cells, which are the Phoenix Flow Systems' Post UV irradiated cells with breaks tailed with 5-BrdU in the presence of TdT; Wash Buffer, Part Number ASWB15; Reaction Buffer, Part Number ASRXB16; TdT Enzyme, Part Number ASTD17; and Br-dUTP, Part Number ASBU18. ReactiBind Biotin Coated Microwell Strip Plates were obtained from Pierce Chemical (Rockford, Il, item no. 15151). White, "U" bottomed, 96 well, microtiter plates were obtained from Thermo Electron Corp. (Franklin, MA, part no. 7105). Aminosilane treated slides (Silane-Prep Slides) were obtained from Sigma (St. Louis, MO, product number S4651). Clearium Mounting Medium was obtained from Surgipath Medical Industries Inc. (Richmond IL). Parafilm 4 in. x 125 ft. roll (Laboratory Film) was obtained from Pechiney Plastic Packaging (Menasha, WI, part no. PM-996). 1.5 mL Eppendorf Tubes were obtained from Fisher Scientific (Cat. No. 22 36 320-4).

2.2. Solutions

TBS-Azide is an aqueous solution which contains in 1 liter: 10 mmol of TRIS, 150 mmol of NaCl, and 7.7 mmol of NaN_3. $Gd(TTFA)_3$-EtOH is a solution that contains 300 μmol of $Gd(TTFA)_3$ per liter of ethanol. BSA pH 7.0 and BSA pH 8.5 are solutions that contain 0.5% w/v BSA in TBS-Azide, with respective final pH values of 7.0 and 8.5. Bicarbonate coupling buffer is a 150 mM NaCl and 20mM $NaHCO_3$ aqueous solution, with a pH of 8.1. Gd Rinse Buffer is based on the Phoenix Flow Systems rinse buffer[6] and consists of: 10 mM TRIS, 150 mM NaCl, 0.1 mM $GdCl_3$, 0.25% (w/v) gelatin, 7.7 mM NaN_3 and 0.1% v/v Triton X-100. The pH was adjusted to 7.4 with HCl. The 5% PEG-EtOH solution is an ethanolic solution containing 50 grams of PEG 1,450 in 1 liter.

2.3. Preparation of Special Materials

2.3.1. BSA Blocked, Biotin Coated Microwell Strips

Excess binding sites of the biotinylated microwells were blocked by the addition of 100 μL of BSA pH 8.5, which was removed by aspiration. This was followed by the addition of a second 100 μL of BSA pH 8.5. The biotinylated wells were then incubated for 15 minutes at room temperature (26 °C) and the supernatant was removed by aspiration. Finally the biotinylated wells were rinsed twice with 100 μL BSA pH 7.0.

2.3.2. Centrifugal Cytology

A pair of Leif Centrifugal Cytology Buckets[7] (Newport Instruments) that fit a Beckman Coulter (Brea. CA) model GPR centrifuge, each of which holds 2 inserts, were assembled with aminosilane treated slides. Four chamber inserts were used. The cells were centrifuged at 300 g for 5 minutes in Leif Buckets and the supernatant was removed by aspiration. One hundred μL of 5% PEG-EtOH solution was added to the fixative inlet of the centrifugal cytology sample chambers and sedimented onto the slide-attached cells by centrifugation. The supernatant was then removed by aspiration. The slides were removed from the Leif Buckets, rinsed twice with ethanol and air dried.

2.3.3. Preparation of EuMac-di-NCS Stained Cells

One mL of HL60 (non-apoptotic) cell suspension (approximately 1 x 10^6 cells in 1 mL) was transferred to a 1.5 mL Eppendorf Tube, centrifuged at 300 g for 5 minutes, and the 70% (v/v) ethanol supernatant was removed by aspiration. The resulting cell pellet was washed with 0.5 mL of TBS-Azide, centrifuged, and aspirated as before. The cell pellet was further washed with 0.5 mL of bicarbonate coupling buffer, centrifuged, and aspirated as before. The cell staining with EuMac-di-NCS was performed by first resuspending the cell pellet with 100 μL of the bicarbonate coupling buffer; this was achieved by pipetting up and down in a 200 μL pipette tip, followed by the addition of 10 μL of 1 M $NaHCO_3$ pH ~8.1. Ten μL of a DMSO solution of EuMac-di-NCS (3-5 mg/mL) was then added and mixed by pipetting. The

EuMac-di-NCS coupling solution was incubated at room temperature for 30 minutes in the dark. The reaction was quenched by addition of 5 μL of 1.5 M NH_2OH pH 8.5 and incubating at room temperature for an additional 15-20 minutes. The cells were then washed three times by addition 0.5 mL TBS-Azide, centrifugation, and subsequent aspiration of supernatant. Fixed dispersions of the cells were prepared by Centrifugal Cytology (Section 2.3.2). The cell monolayer was flooded with 2 drops of 300 μM $Gd(TTFA)_3$ in ethanol and air dried. The slide bound cells were rinsed twice with ethanol to remove the excess dried LEL precipitate from the slide. Thirty μL of Clearium Mounting Medium was pipetted onto the cell area, making sure that all cells were covered. The solvent was removed from the Clearium by mild heat generated with a heat gun.

2.3.4. Preparation of DAPI Stained Cells

One half mL of a HL60 (non-apoptotic) cell suspension (approximately 1 x 10^6 cells in 1 mL) was used for the centrifugal cytology preparations (Section 2.3.2). The cell monolayer was rehydrated by submerging the slide in TBS Azide pH 7.4 in a 50 mL centrifuge tube for 5 minutes. Excess liquid was removed with Kimwipes and the cells were stained with 100 μL of 10 μM DAPI solution in TBS Azide pH 7.4 for 5 minutes. The cells were rinsed with TBS Azide by flooding the monolayer several times. The DAPI stained cells were dried by flooding the cell area with ethanol and allowing the solvent to evaporate to dryness. Two drops (~25 μL) of LEL were applied and the ethanol was allowed to evaporate. The slide was rinsed twice with ethanol to remove the excess of dried LEL. A thin layer of Clearium was applied to the slide and the solvent was removed from the Clearium by mild heat generated with a heat gun.

2.4. General Procedures, Equipment, and Instruments

2.4.1. CCD Camera

Images were obtained with a Peltier cooled, monochrome Quantitative Imaging Corp. (Burnaby, BC, Canada) Retiga-1350 EX, 12 bit ADC, CCD camera (1280 x 1024). According to the manufacturer's specification, this camera operates at 25°C below ambient temperature, or ca. 0°C. The gray levels of the images were inverted for display. Darkness indicates strong luminescence.

2.4.2. Microwell Measurements

An Ultraviolet Products (UVP) (Upland, CA) Epi Chem II Darkroom was equipped with a 619 nm narrow-band, 5.6 nm half-width, emission filter (Omega 618.6NB5.6) and a special adaptor to mount the Retiga-1350 EX camera. The microtiter strips were inserted into UVP Epi Chem II Darkroom and illuminated with the long UV (ca. 365 nm) bulb; the emission was passed through the 619 nm emission filter. Digital images of the strips were acquired with the Retiga-1350 EX camera. The image of the center 81% of the area of the well was analyzed. The mean of the luminescence emission intensity was calculated with Fovea PhotoShop (Reindeer Games, Inc. Asheville, NC) plug-in under Filter/IP*Features/Regions.

2.4.3. Microscope

A Leitz MPV II fluorescence microscope equipped with a 10X 0.25 NA, a 40X 0.65 NA, and an infinity corrected objective high ultraviolet transmission UPL Fluorite 60 oil NA 1.25 with aperture (Olympus Part# 1UB532) was employed to observe and to electronically photograph the cells. UV and blue illumination was provided by a 100 watt Mercury-Xenon short arc. The UV fluorescence was excited at 365 nm and the emitted light was observed through an Omega Optical (Brattleboro, VT) PloemoPak cube UV DAPI, equipped with a 365 nm narrow-band-width excitation filter (Omega 365HT25) and a 400 nm Beamsplitter (Omega 400DCLP02). The CCD optical path was optionally equipped with either a 619 nm narrow-band emission filter (Omega 618.6NB5.6) or a standard DAPI 450 nm emission filter (Omega 450DF65).

2.4.4. Image Manipulation

The TIFF images produced by the Retiga-1350 EX camera were manipulated with Adobe® (San Jose, CA) Photoshop® 7.0. All images were transformed into 8 bit grayscale and inverted to facilitate visualization. The conversion of a white image on a black background to a black image on a white background produces the equivalent of a conventionally stained absorbance image. This was preferred because it is familiar to practitioners in the field of cytology, such as cytotechnologists and pathologists. Other manipulations of 8 or 16 bit images were performed with Fovea (Reindeer Games, Inc. Asheville, NC).

2.4.5. Confocal Microscope

A Leica TCS-SP1 was used for the one-photon studies. The machine was aligned and calibrated for proper performance as previously described[8]. The cells were excited with approximately 10 mw 365 nm laser light using a dichroic reflector. A Plan Apo 63x water immersion lens was used (NA 1.2). Using the lambda scan feature, spectra from 450 nm to 700 nm were obtained in 5 nm increments using PMT 2, which was determined to be the most accurate PMT in the machine. A small circular ROI was placed in each cell and the intensity was recorded. The resultant curve is a spectrum of intensity vs. wavelength.

2.4.6. Nonlinear Optical Microscopy

A Zeiss LSM 510/NLO/Combi instrument, mounted on an Axiovert 200 and equipped with a Coherent Mira femtosecond titanium-sapphire laser pumped with an 8W Verdi laser, was used for two-photon microscopy. The laser output was tuned to 800 nm, and the specimen imaged using a C-Apochromat 40 x 1.2 N.A. water immersion objective. To reduce the contribution from adjacent pixels, the image was collected through the descanned pathway, using a confocal aperture equivalent to one Airy unit.

3. DATA

3.1. Linearity Study of EuMac-Streptavidin Binding to Biotinylated Microwells

A series of dilutions of the EuMac-Streptavidin conjugate were made to produce concentrations of 1.92, 9.6, 48, 240, and 1,200 ng/mL in BSA pH 7.0. Two solutions, one containing 150,000 ng/mL of streptavidin and the other containing 150,000 ng/mL of the fluorescein conjugate of streptavidin, both in BSA pH 7.0, were also prepared. One hundred µL of each of these solutions was added to a well in a BSA-blocked, biotin-coated microwell strip and the solutions were mixed by lightly tapping against the microtiter strip wall. The microwell strip was covered with Parafilm to prevent evaporation and incubated at room temperature (26 °C) for 30 minutes in the dark. The supernatants were removed and the biotinylated wells were washed 3 times with 100 µL of BSA pH 7.0, which was removed by aspiration. The biotinylated wells were then allowed to dry. Two drops (~25 µL) of the $Gd(TTFA)_3$-EtOH were added to each biotinylated well. The microtiter strips were allowed to air dry overnight in the dark.

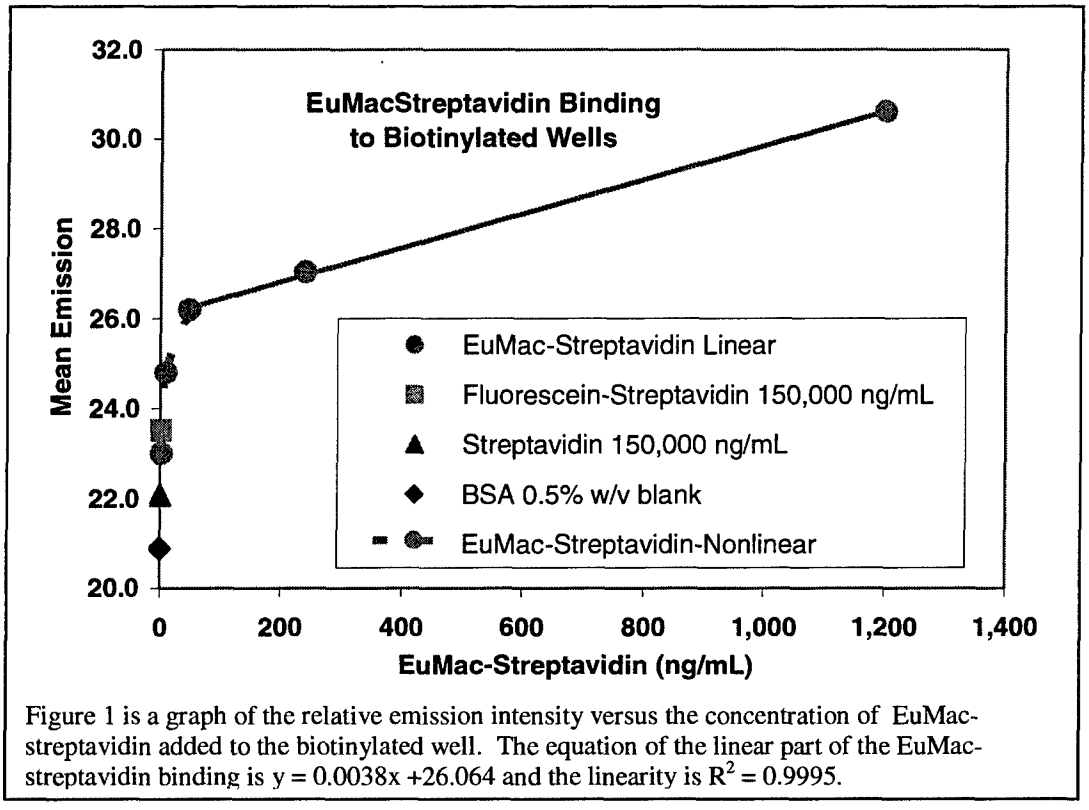

Figure 1 is a graph of the relative emission intensity versus the concentration of EuMac-streptavidin added to the biotinylated well. The equation of the linear part of the EuMac-streptavidin binding is $y = 0.0038x + 26.064$ and the linearity is $R^2 = 0.9995$.

Figure 1 shows that the EuMac-tagged member of a specific combining pair can be detected and quantitated after being dried from a homogeneous solution. For unknown, possibly instrumental reasons, the first two points have a much steeper slope. The emissions of control samples of streptavidin and fluorescein-labeled streptavidin, both at 150,000 ng/mL, and of BSA at 5 mg/mL, are shown at the ordinate. Although the fluorescein conjugate has saturated the well, only a very small part of the long wavelength tail of the emission passes through the 619 nm filter. The sensitivity of this assay can be improved by the use of either a time gated system[9] and or an optimized optical system capable of gathering a larger part of the luminescence emissions.

3.2. Optimization of the Lanthanide Enhanced Luminescence

$Gd(TTFA)_3$ was added to ethanol to produce a 1.0×10^7 nM (1.0×10^{-2} M) stock solution. This solution was serially diluted tenfold with ethanol, to produce a series of solutions, $Gd(TTFA)_3$-EtOH solutions, with the lowest concentration being 1.0 nM (1.0×10^{-9} M). HTTFA was added to ethanol to produce a 30 mM stock solution. This solution was serially diluted tenfold with ethanol, to produce a series of solutions, HTTFA-EtOH solutions, with the lowest concentration being 3.0 nM (3.0×10^{-9} M). Each HTTFA-EtOH solution had the same HTTFA enhancer content as its corresponding $Gd(TTFA)_3$-EtOH solution.

Just prior to use, the 0.05 mg/mL EuMac-Streptavidin solution was diluted with the BSA pH 7.0 solution to a final concentration of 0.5 μg/mL. The EuMac-Streptavidin solution (50 μL of the 0.5 μg/mL solution) was pipetted into each of 16 BSA-blocked biotin-coated microwells. An equal number of control wells did not receive the EuMac-Streptavidin. The microtiter strip was covered with Parafilm to prevent evaporation and incubated at room temperature (25 °C) for approximately 30 minutes in the dark. The supernatants were removed and the EuMac-Streptavidin and control microwells were washed 3 times with 100 μL of BSA pH 7.0, which was removed by aspiration. The wells were then allowed to dry at room temperature. Thirty μL of the $Gd(TTFA)_3$-EtOH serial dilutions, starting at 1.0×10^7 nM, were sequentially added to 16 biotinylated wells; eight of which had been previously treated with the EuMac-Streptavidin solution. Thirty μL of the control TTFA-EtOH serial dilutions, starting at 3.0×10^7 nM, were also sequentially added to 16 other biotinylated wells; eight of which had been previously treated with the EuMac-Streptavidin solution. The microtiter strips were allowed to air dry overnight in the dark.

Four sets of data were measured: HTTFA-EtOH added to eight control wells, HTTFA-EtOH added to eight EuMac-Streptavidin coated wells, $Gd(TTFA)_3$-EtOH added to eight control wells, and $Gd(TTFA)_3$-EtOH added to eight EuMac-Streptavidin coated wells.

The data are shown in Table 1. The results for the wells treated with the $Gd(TTFA)_3$ solutions are shown on the left and those for the wells treated with the HTTFA solutions on the right. The results for the HTTFA control wells are essentially constant; whereas, the luminescence of the $Gd(TTFA)_3$ control wells increases with concentration, as expected because of the small europium contamination in the gadolinium. For both solutions, the maxima occurred at the second highest concentration, 1.0×10^6 nM $Gd(TTFA)_3$ and 3.0×10^6 nM HTTFA. An inner filter effect is one explanation for the quenching of the luminescence at the highest concentration.

Table 1, Luminescence Emissions from the Air-Dried Wells

$Gd(TTFA)_3$ (nM)	EuMac-Strep + $Gd(TTFA)_3$	$Gd(TTFA)_3$		HTTFA (nM)	EuMac-Strep + HTTFA	HTTFA
1.0×10^7	140	61		3.0×10^7	42	22
1.0×10^6	160	40		3.0×10^6	49	23
1.0×10^5	75	37		3.0×10^5	46	24
1.0×10^4	31	25		3.0×10^4	35	23
1.0×10^3	25	26		3.0×10^3	31	23
1.0×10^2	23	25		3.0×10^2	28	23
1.0×10^1	21	21		3.0×10^1	25	22
1.0	19	20		3.0	22	20

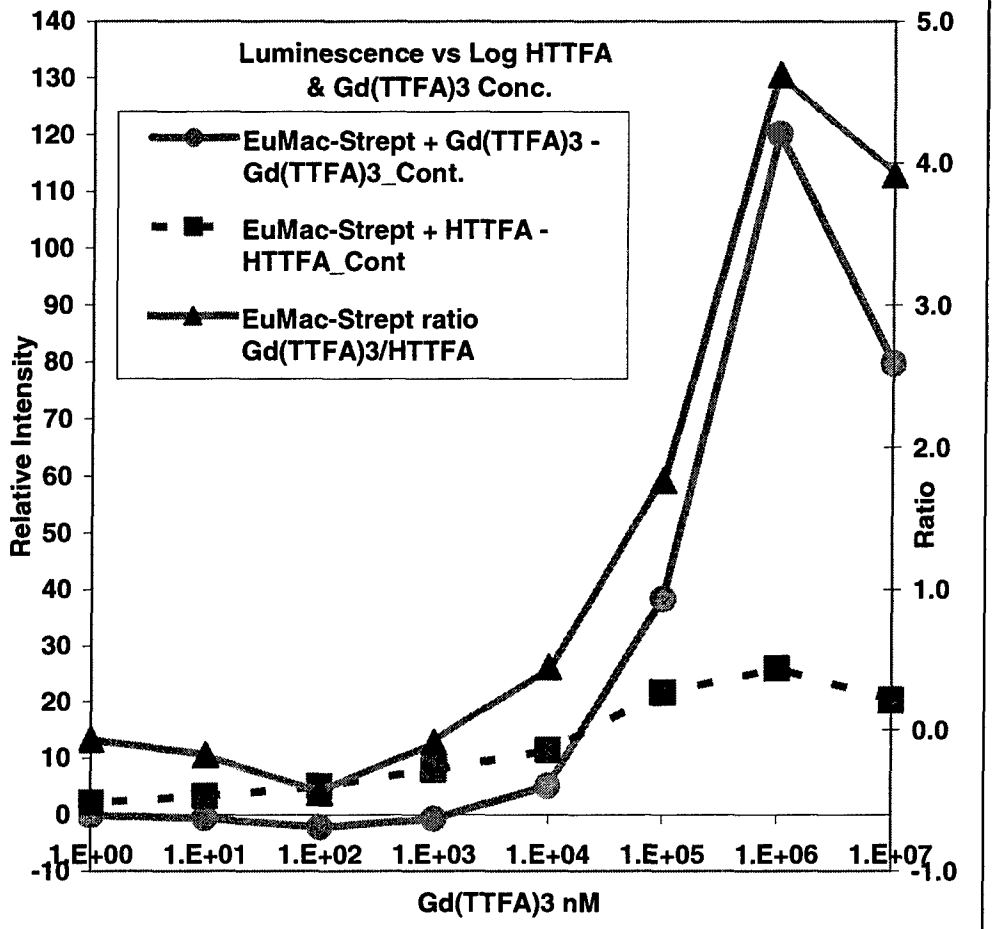

Figure 2 is a plot of relative net luminescence vs. $Gd(TTFA)_3$ and HTTFA concentrations.

Figure 2 consists of plots of the net luminesce of the EuMac-Streptavidin stained wells, corrected for the background from the control wells. Only the concentration of the $Gd(TTFA)_3$ is shown. In each sample the concentration of the HTTFA solution is three times that of the $Gd(TTFA)_3$, in order to maintain a constant number of available TTFA ligands. The figure shows the increases in luminescence resulting from the additions of the $Gd(TTFA)_3$ (circles) and the HTTFA (squares) solutions. The ratio (triangles) of this increase is also shown. At low concentrations of $Gd(TTFA)_3$, the luminescence of the EuMac-Streptavidin wells was lower than that of the wells with comparable concentration of HTTFA. This was possibly due to a number of the europium macrocycles being incompletely complexed with TTFA because of the competition between Gd(III) and Eu(III) for the available TTFA ligands. At higher concentrations, the supply of TTFA ligands from the $Gd(TTFA)_3$ was sufficient to produce the LEL effect, which was maximum at $1.0x10^6$ nm with a ratio of 4.6.

This experiment was repeated (data not shown) with a different lot of the EuMac-Streptavidin. The maxima for both sets of wells occurred again at the second highest concentration, $1.0x10^6$ nM $Gd(TTFA)_3$ and $3.0x10^6$ nM HTTFA. The maximum ratio of the emissions with and without gadolinium ion was again 4.6 and occurred again at the second highest concentration. For both experiments, removal of the europium contaminant from the gadolinium would significantly increase this ratio by decreasing the emissions from the $Gd(TTFA)_3$ controls.

3.3. Preparation of EuMac-anti-5-BrdU Directly Stained S Phase Cells

The protocol[6] of the Phoenix Flow Systems (San Diego, CA) ABSO-LUTE-S™ kit was followed with the substitution of the EuMac-Anti-5-BrdU for the fluorescein labeled antibody, and the use of the fluorescein labeled antibody as a control. This direct staining procedure was based on the SBIP™ (Strand Break Induced Photolysis) technique[10]. Parallel flow cytometry measurements with fluorescein labeled anti-5-BrdU permitted direct quality control for all reagents except the EuMac-mono-NCS labeled anti-5-BrdU. Parallel centrifugal cytology preparations were made with the fluorescein labeled antibody. The cells were allowed to air-dry from the ethanol, because the low surface tension of ethanol produces minimal morphological distortion.

Briefly, this protocol started with photolysis of the BrdU labeled DNA and tailing by the addition of 5-BrdU with terminal deoxytransferase. Subsequently, the cells were washed twice by centrifugation for 5 minutes with 0.5 mL of Gd Rinse Buffer, and the supernatant was removed. The cell pellet was resuspended in 0.1 mL of 40 μg/mL of EuMac-anti-5-BrdU; the tubes were wrapped with aluminum foil and incubated in the dark for 30 minutes at room temperature. After the 30 minute incubation, 0.5 mL of Gd Rinse Buffer was added to the staining solution. The cell suspension was centrifuged and the supernatant removed, as before. The wash with Gd Rinse Buffer was repeated. Fixed dispersions of the cells were prepared by Centrifugal Cytology (Section 2.3.2). The cell monolayer was flooded with 2 drops of 134 μM $Gd(TTFA)_3$ in ethanol and air dried. The slide-bound cells were rinsed twice with ethanol, removing excess liquid each time, and air dried. Thirty μL Clearium Mounting Medium was pipetted onto the cell area, making sure all cells were covered. The solvent was removed from the Clearium by mild heat generated with a heat gun.

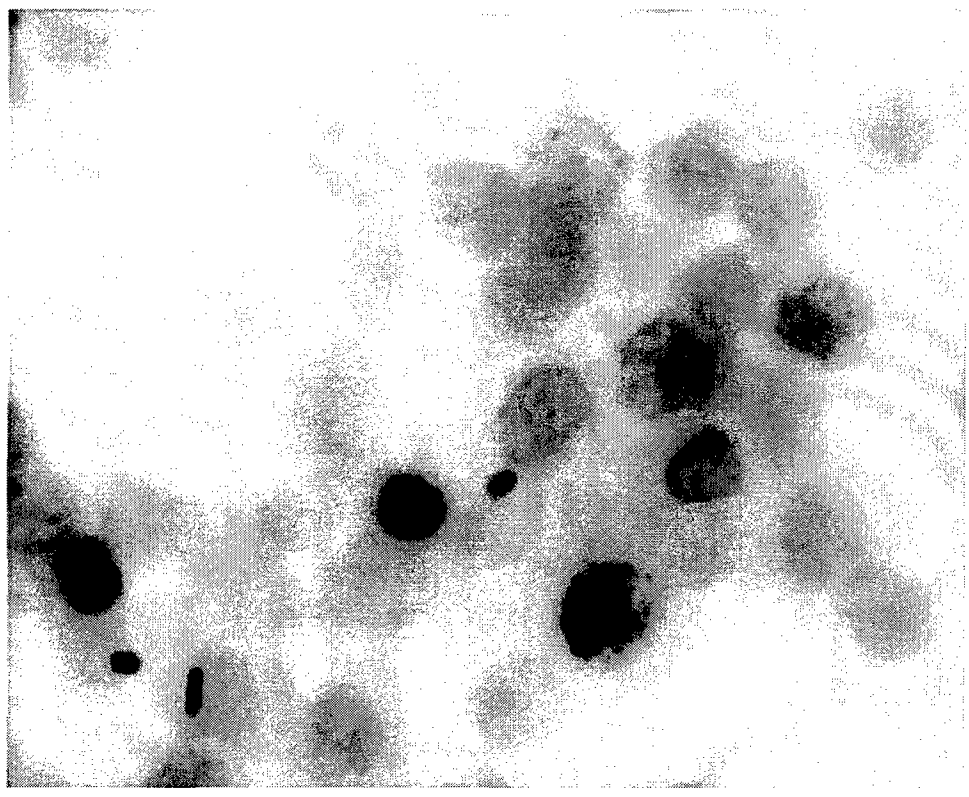

Figure 3 is an inverted image of the 619 nm emission of the EuMac-anti-5-BrdU stained cells in S phase, produced with a 60 x oil immersion lens. The continuous excitation was at 365 nm and the exposure was for 30 seconds. The image of the cells was binned to 680 x 518 pixels.

Figure 3 was slightly overexposed to show the unlabeled cells and the small dark granules in some of the cells, which are the islands of initial DNA synthesis. The labeled rod-like structures are probably bacteria. As demonstrated by this detection of S phase, it is now possible with only minimal changes from current protocols to obtain preparations of cells directly stained with europium-tagged antibodies, and to intensify their emission by the LEL effect produced with a stable ethanol solution.

3.4. Confocal Microscopy

3.4.1. Two Photon Excitation of the EuMac Labeled Cells Dried from a $Gd(TTFA)_3$ Solution

A standard glass microscope slide with EuMac-di-NCS labeled cells was prepared by first staining the cells in suspension (Section 2.3.3), followed by Centrifugal Cytology (Section 2.3.2). In order to facilitate confocal microscopy, a very thin layer of Clearium was pipetted onto the cells, covering the entire cell area which was then coverslipped. The slides were examined with a laser scanning LSM510 NLO/Combi system Zeiss confocal microscope equipped with a Coherent Mira Ti-sapphire laser and a C-Apochromat 40 x 1.2 N.A. water immersion objective. The excitation was at 800 nm, and the emission between 510 and 685 nm was detected. Each pixel was 0.15 by 0.15 nm and the scanning dimensions were both 76.8 nm. A 90 μm pinhole, which corresponds to one airy unit, was used. The laser pulse width was 100 fs and the pulse rate was 76 MHz (every 12 ns), which should produce about 500 pulses in the 6.4 μsec dwell time on the pixel. Each line was scanned four times and the results were averaged. The optical zoom was 3. The image was displayed with the Zeiss LSM5 image browser and copied into Adobe Photoshop where it was transformed into grayscale and inverted.

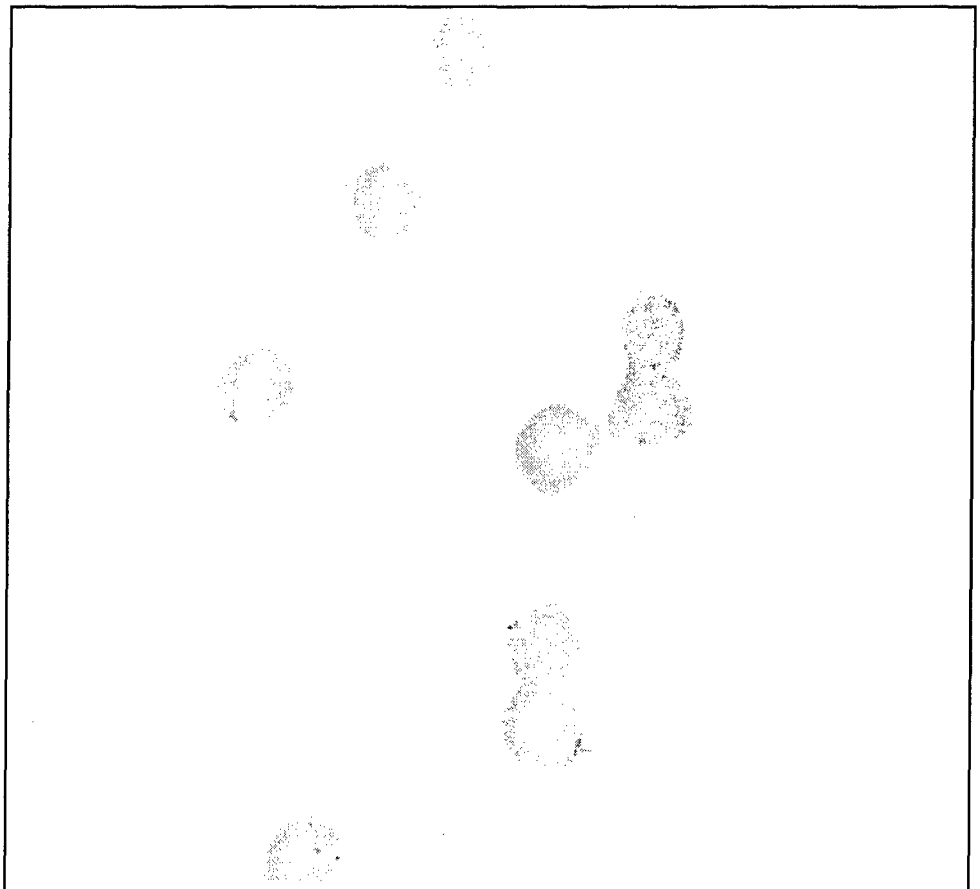

Figure 4 is an inverted image of two-photon excited EuMac-di-NCS stained cells.

Figure 4 has minimal background, showing that the signal to noise ratio was excellent. This result was unexpected; since the lifetime[11] of the EuMac in the LEL micellar suspension is 955 μsec and the dwell time was 6.4 μsec, approximately 1.2% of the emitted light should have been detected. Since this image was of an unfiltered emission, a significant part was from the background blue emission of the dry $Gd(TTFA)_3$. Examples of this background blue emission are shown in Figure 5. There is also the possibly, that in the solid state either the lifetime or the quantum efficiency of the two-photon emission, or both, may differ greatly from those of the LEL micellar suspension. In any event, these experimental results support the importance of investigating the use of lanthanide tags with emission enhancement by a second lanthanide, LEL, for two-photon laser scanning confocal microscopy. Since the long wavelengths used for excitation are minimally scattered by biological materials, the detection of analyte-binding species located significantly below the surface of the sample will be possible, particularly when red or near infrared emitting lanthanides are used.

3.4.2. Spectral Studies of UV Excited Emissions

Two cell dispersions on standard glass slides were prepared by centrifugal cytology (Section 2.3.2). The spectra shown in Figure 5 and Figure 6 were obtained with a Leica Confocal microscope TCS-SP1 equipped with a lambda scan feature. Using this feature, a spectrum was obtained of a field containing 4 cells by taking 50 sections in 5 nm increments between 450 nm and 700 nm. A small ROI was made in each of the cells and the spectral was recorded. Note that three of the cells have similar emission intensities between 450 nm and 575 nm, while one cell shows minimal emission in this spectral region.

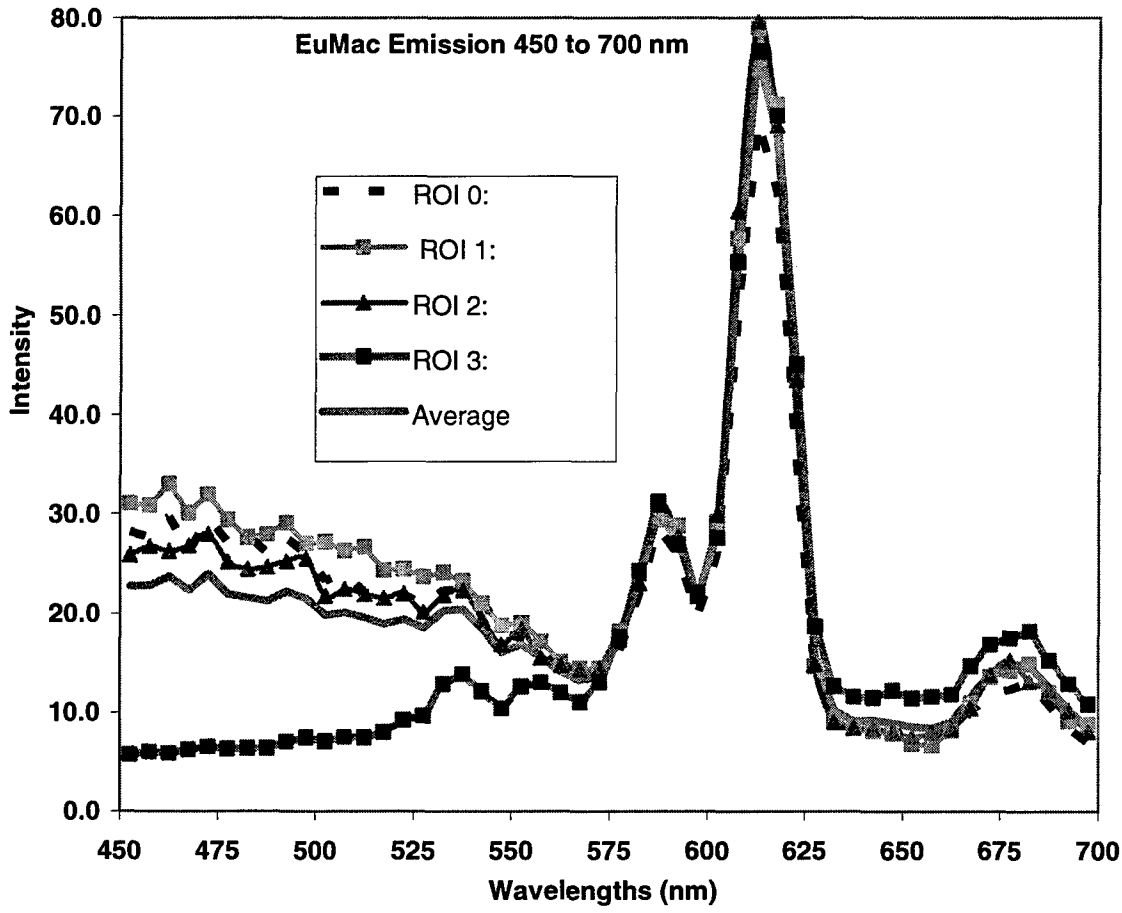

Figure 5 shows emission spectra of 4 regions of interest representing 4 cells and their average. The solid line without symbols is the average. The excitation was at 365 nm and the emission resolution is a nominal 5 nm.

All of the cells have identical profiles in the region between 575 and 625nm. The spectra of the regions of interest were acquired with the manufacturer's software and were graphed using Microsoft® Excel. All the EuMac spectra and their average are similar to those previously published. However, the spectra are shifted approximately 5 nm to shorter wavelengths, and the maximum peak of the average at half maximum has a width of 19 nm, compared to the previously reported[12] 5.2 nm value. Even with this instrumental broadening, the europium emission is very narrow compared to that of the conventional dye, DAPI, shown in Figure 6.

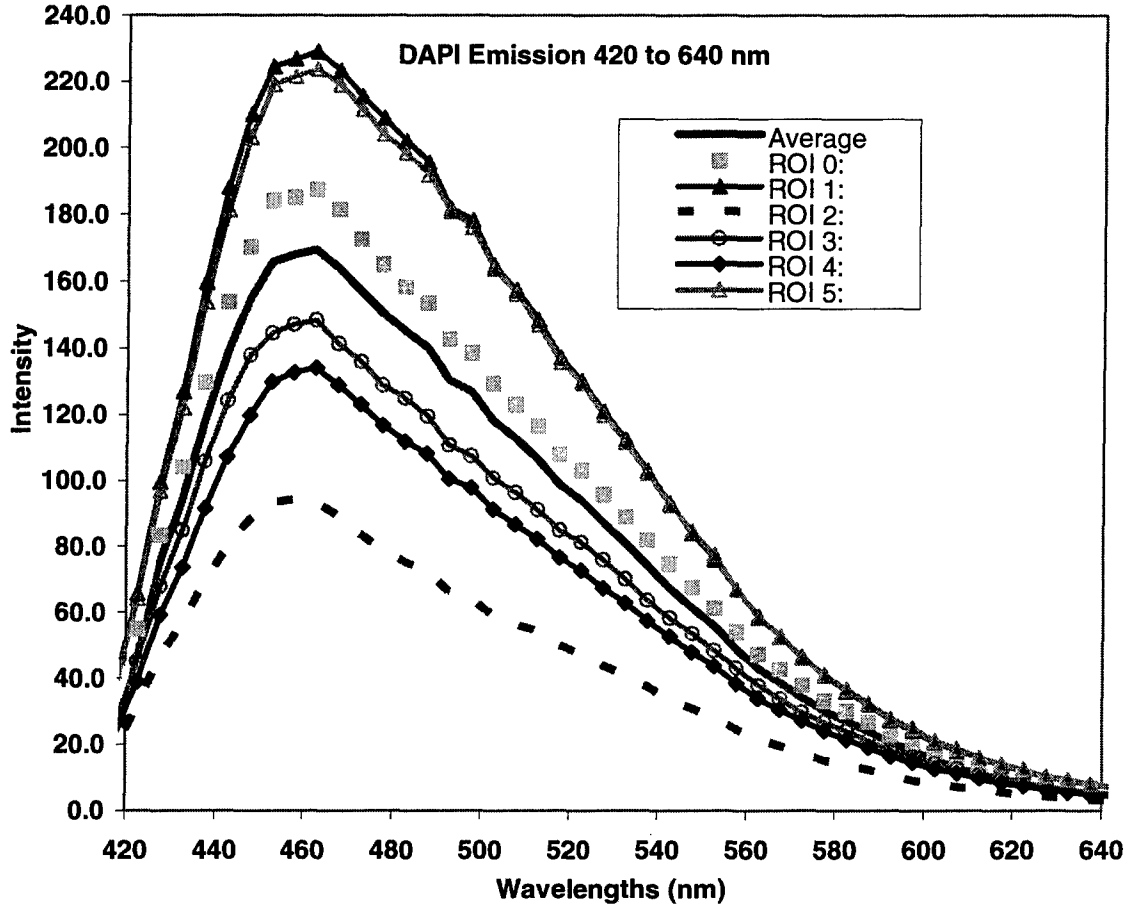

Figure 6 shows the emission spectra between 420 nm and 640 nm of 5 different cells and their average. The solid line without symbols is the average. The excitation was at 365 nm and the emission resolution is a nominal 5 nm.

3.5. A Common Enhancer for Both Europium and Terbium Quantum Dyes

Preliminary studies indicate the possibility of using 2,6-pyridinedicarboxylic acid (2,6-PDCA) to enhance simultaneously the luminescence of both the europium and terbium Quantum Dyes. Four ethanol solutions were prepared: 0.1 mol/L 2,6-PDCA enhancer, 0.1 mol/L NaOH, 0.01 mol/L unfunctionalized europium macrocycle, EuMac-un; and 0.01 mol/L unfunctionalized terbium macrocycle, TbMac-un. Thirty µL of both the 2,6-PDCA and the NaOH solutions were pipetted into each of 8 wells of a white, "U" bottomed, 96 well, microtiter plate. One set of 4 wells was treated with the EuMac-un solution (100 µL added to each well); the other set of 4 was treated with the TbMac-un solution (100 µL added to each well). The solutions were allowed to dry overnight. The luminescence emission spectra of the solid residues, each of which contained 1 µmole of lanthanide macrocycle, were obtained with a Varian Cary

fluorometer operated in time-gated luminescence mode with a delay of 100 μsec. Fifty emission spectra were summed to produce the final spectrum. The excitation and emission slits were respectively 10 and 2.5 nm.

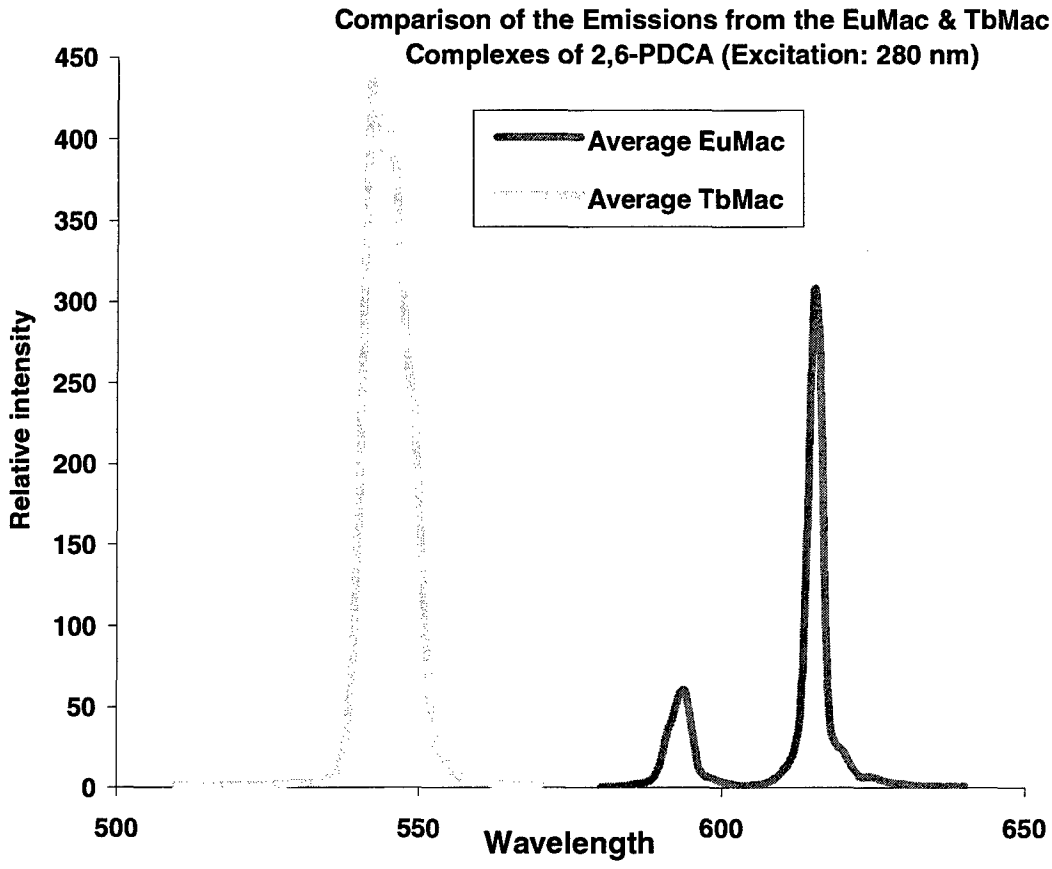

Figure 7 shows the emission spectra of the unfunctionalized terbium (left) and europium (right) macrocycles. The PMT voltage was 600.

In order to maximize the emission signals shown in Figure 7, both the europium and the terbium macrocycles were excited at 280 nm. Previously, the use of the special fused silica objectives that transmit this short wavelength would have been prohibitively expensive. However, the use of this and much shorter wavelengths in the manufacture of integrated circuits has now greatly increased the feasibility of their use in cell biology. The spectra of the europium and the terbium macrocycles do not overlap and can be time-gated from the DAPI emission. Thus it is now possible to have two background-free ratiometric labels that can be used for fluorescence in situ hybridization, FISH, and comparative genomic hybridization[13]. The exciting possibility exists that this combination could be employed with present two photon excitation systems.

4. RESULTS AND CONCLUSIONS

A simple, stable lanthanide-enhanced luminescence solution has been developed, tested, and its use has been optimized for dry preparations including microtiter plates and cell dispersions. Since the cell preparation includes air drying from a plastic medium, the light induced fading can be minimized[9]. Surprisingly, in spite of the mismatch between the short dwell time of commercial confocal microscopes and the long lifetimes of the lanthanides, it has been possible to obtain one and two photon images and even to measure the spectrum produced by single photon illumination. Although, it has not been established what part of the emission is actually from the two photon excitation of the lanthanide ion, it has

been established that the two-photon excitation of the first member of the LEL energy transfer complex is efficient. The amorphous mixture of organic-biological materials, lanthanide macrocycle label, and salt of the second lanthanide and organic anion enhancer appears to possess electro-optical properties similar to those of lanthanide phosphors, but in addition offer the advantage of small size tag and the opportunity to change and optimize the composition. The surprising efficiency of the two-photon excitation of lanthanide complexes (upconversion), its mechanism for complexes in solution, and the utility of this process for background-free measurements has recently been described[14]. Time-gating and increasing the dwell-time of the confocal microscope will also significantly increase the capacity to detect two-photon excitation of lanthanide luminescence.

Preliminary results indicate that both the europium and the terbium macrocycles can utilize 2,6-pyridinedicarboxylic acid as a common luminescence enhancer. Since these results agree with Raymond's group[15] finding that excitation between 350 to 360 nm produced strong emission from the europium and terbium complexes of phthalamide derivatives, it can be concluded that there may be a significant number of other possible enhancers for the simultaneous measurement of the luminescence emissions of two lanthanide complexes.

Although a single inorganic particles, such as a phosphor or Quantum Dot, may emit more intensely than a single lanthanide macrocycle under comparable excitation flux, the lanthanide macrocycle labels reported here have the following advantages: 1) Multiple lanthanide macrocycles attached to a polymer carrier[1,16] can be contained in a volume equal to that of a single inorganic particle; and their combined emission intensity is proportional to their number, since they do not suffer from concentration quenching. 2) The LEL effect permits various species, other than those directly bound to the lanthanide ion, to act as photon traps and energy-transfer donors. 3) The lanthanide macrocycles do not interfere sterically with the binding of antibodies and other biomolecules or interfere with intracellular staining. 4) The lanthanide macrocycles have the narrowest emission known, 5.2 nm at half maximum[12]. And 5) This emission is based on quantum states and thus does not change from batch to batch.

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